ENZYMIC CONVERSION OF STARCH

PREPARATION OF AN IMMOBILIZED TWO-ENZYME SYSTEM, β-AMYLASE-PULLULANASE, TO AN ACRYLIC COPOLYMER FOR THE CONVERSION OF STARCH TO MALTOSE

KAJ MÅRTENSSON

LIVSMEDELSTEKNOLOGI
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av

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To Ann-Margreth and Patrik

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This thesis is based on the following communications, referred to in the text by their Roman numerals:

I K. Mårtensson, On the Preparation of Pullulan from *Pullularia pullulans*, AUL:s halvårsskrift, in press, 2, (1974).

II K. Mårtensson, Preparation and Purification of Pullulanase from *Aerobacter aerogenes*, AUL:s halvårsskrift, in press, 2, (1974).

III K. Mårtensson and K. Mosbach, Covalent Coupling of Pullulanase to an Acrylic Copolymer Using a Water Soluble Carbodiimide, Biotechnol. Bioeng., 14, 715 (1972).

IV K. Mårtensson, Preparation of an Immobilized Two-Enzyme System, β-Amylase-Pullulanase, to an Acrylic Copolymer for the Conversion of Starch to Maltose. Part I: Preparation and Stability of Immobilized β-Amylase, Biotechnol. Bioeng., in press, 16, 000 (1974).

V K. Mårtensson, Preparation of an Immobilized Two-Enzyme System, β-Amylase-Pullulanase, to an Acrylic Copolymer for the Conversion of Starch to Maltose. Part II: Co-Coupling of the Enzymes and Use in a Packed Bed Column, Biotechnol. Bioeng., in press, 16, 000 (1974).

VI K. Mårtensson, Preparation of an Immobilized Two-Enzyme System, β-Amylase-Pullulanase, to an Acrylic Copolymer for the Conversion of Starch to Maltose. Part III: Process Kinetic Studies on Continuous Reactors, Biotechnol. Bioeng., in press.

INTRODUCTION

Enzymes in the starch industry

Enzymes have been available for many years for the hydrolysis of starch, but due to the high cost and the lack of knowledge about the enzymic processes, acid conversion has been the main method for starch degradation. During the last fifteen years major advances in this field of enzyme technology have been made, and today hydrolytic processes with the aid of α-amylase, β-amylase and amyloglucosidase are well established[1,2]. A typical enzyme process for the manufacture of a starch syrup includes one liquefaction step, where the starch molecules are partially hydrolyzed and brought into solution with the aid of an acid or bacterial α-amylase, followed by one saccharification step, where the liquefied starch is converted further into various sugar fragments by the use of suitable combinations of hydrolytic enzymes. In this way glucose or maltose syrups of different dextrose equivalents (DE, content of reducing sugars calculated as glucose in percentage of the dry substance) are manufactured with the aid of bacterial or fungal amylases, malt enzymes or amyloglucosidase of microbial origin. New approaches in starch enzyme technology have been made with the use of glucose isomerase[3], for the conversion of glucose to fructose, and with the use of debranching enzymes[4] for the production of short chain amylose[5,6] and high purity maltose[7,8].

Debranching enzymes

Debranching enzymes, *i.e.* enzymes which specifically split the α-1,6-glucosidic linkages in starch-type polysaccharides, of industrial potential include pullulanase[9] and isoamylase[10], which are both obtained from microbial sources. Tables I and II summarize some proposed applications of debranching enzymes and potential uses of the products obtainable by starch hydrolysis.

Table I. Potential applications of debranching enzymes.

a. Analytical tool in elucidation of polysaccharide structures[11,12]
b. Production of low molecular weight amylose from starch[5,6]
c. Production of high purity maltose from starch[7,8]
d. Preparation of low viscosity starch solutions[13]
e. Production of maltotriose from pullulan[14]
f. Use in brewing from unmalted cereals[15]

Table II. Potential uses of low molecular weight amylose and high purity maltose[16].

Low molecular weight amylose

a. Production of transparent, edible coatings
b. Use as a tablet binder
c. Sizer for textiles and fabrics

High purity maltose

a. Confectionary industry, especially in hard candies
b. Beer production by direct fermentation of the hydrolyzate
c. Pharmaceutical industry, production of vaccines and antibiotics
d. Hydrogenation to maltitol, a non-digestible sweetener

Syrups having a relatively high maltose content are by conventional methods produced with the aid of malt enzymes, giving an upper limit for economical starch saccharification of DE 50–55, with a maltose content of about 60%[2]. Such syrups contain a considerable amount of undesirable glucose and small branched dextrins. By instead using a combination of β-amylase and a debranching enzyme (Figure 1), it is possible to convert starch to a syrup with a maltose content greater than 90% with still a relatively small amount of glucose present[7,8,16]. This high maltose syrup possesses valuable properties, as compared to conventional syrups containing more glucose, because of its less hygroscopic properties and decreased tendency to crystallize.

Fig. 1. Action of β-amylase(→) and a debranching enzyme (⋯▸) on amylopectin.

Immobilized enzymes

A new trend in enzyme technology, which during recent years has resulted in extraordinary activity in academic and industrial laboratories, is research on immobilized enzymes[17-21]. By the immobilization procedure, a continuous catalytic reaction can be applied with the enzyme confined during the process. In Table III different principle methods employed for enzyme immobilization are summarized.

Table III. Principle methods employed for enzyme immobilization.

a. Covalent binding of the enzyme to a polymer
b. Intermolecular crosslinking of the enzyme
c. Adsorption of the enzyme on to a matrix
d. Entrapment of the enzyme in a gel lattice.
   (Often used in combination with a.)
e. Retention of the enzyme by a semipermeable membrane

The most frequently used method, and one of the remaining methods of choice, when macromolecular substrates or products are involved in the catalytic process, is covalent binding of the enzyme to a water-insoluble functionalized polymer. This technique is based upon use of either reactive polymers or activated polymers which are allowed to react with the enzyme, thus resulting in an immobilized conjugate where the enzyme *per se* usually has lost more or less of its catalytic activity. On the other hand, the immobilized preparation offers certain advantages over the free soluble enzyme, some of which are summarized in Table IV.

Table IV. Potential advantages of immobilized enzymes.

a. The enzyme can be reused
b. Permits a continuous mode of operation
c. Permits a rapid termination of the reaction
d. Greater operational flexibility
e. Possibly greater efficiency in multistep reactions
f. May exhibit altered, desired properties
g. Use as model systems of natural, membrane-bound enzymes

The preferred choice of support material and covalent binding procedure is greatly dependent on the specific characteristics of the enzyme and the reaction which it has to catalyze, as well as on the desired application requirements of the immobilized preparation. Immobilized multienzyme systems[22-24], where two or more enzymes, catalyzing consecutive reactions, are bound to the same matrix surface, represent important approaches towards the imitation of biological systems, as well as towards the development of more efficient and complex industrial enzyme processes.

Enzyme reactors

Industrial use of immobilized enzymes may be either in batch or semi-batch treatments, where the enzyme after the reaction can be recovered and reused, or in an enzyme reactor which permits a continuous mode of operation[25-27]. Many different types of enzyme reactors have been presented, of which the two basic types, *i.e.* the stirred tank reactor and the plug flow reactor, have been studied most extensively. Table V shows some of the major advantages or disadvantages with these types of enzyme reactors.

Table V. Comparison of some general properties of a plug-flow reactor (PFR) and a continuous stirred tank reactor (CSTR).

PFR

a. Usually most efficient at low substrate concentrations and high degrees of conversion
b. Seriously affected by a substrate-inhibited reaction
c. Use may be restricted by high pressure drops and by plugging
d. Greater operational flexibility, well-defined residence time

CSTR

a. Usually most effective at high substrate concentrations and low degrees of conversion
b. Seriously affected by a product-inhibited reaction
c. Use may be restricted by requirements of product uniformity or due to the longer residence time
d. Easier catalyst replacement and process control

Among the factors affecting the choice of a certain reactor system are the operational requirements, reactor costs and utilization, the reaction kinetics and the requirement of catalyst replacement of regeneration. The first industrial scale operation employing an immobilized enzyme was achieved in the hydrolysis of acetyl-L-methionine by aminoacylase[28]. Examples of other well-documented, successful uses on a large scale or pilot plant scale include the production of 6-aminopenicillanic acid (6-APA) by hydrolysis of benzylpenicillin with penicillin amidase[29], isomerisation of glucose to fructose with the aid of glucose isomerase[30], and conversion of starch to glucose by use of immobilized amyloglucosidase[31,32].

Aim of the project

The goal of the present investigation has been formulated bearing in mind the potential of starch syrups with a high maltose content and the advantages of immobilized enzymes in industrial applications. The aim has been to develop an immobilization procedure for β-amylase and pullulanase, to make use of the immobilized enzyme system to convert starch to a product with a high maltose content, and to study some of the process parameters of importance from an industrial point of view. This work was also intended to serve as a model study for the way in which enzymes can be immobilized and as such utilized for a technical application, with special reference to the starch industry.

MATERIALS AND METHODS

Materials

1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-p-toluene-sulphonate (CMDI) was purchased from Aldrich Chemical Co., Milwaukee, Wis., Bio-Gel CM 100 (high capacity 100-200 mesh, a crosslinked copolymer of acrylamide-acrylic acid) from Bio-Rad Lab, Richmond, Calif. and Tri Sil silylating reagent was obtained from Pierce Chemical Co., Rockford, Ill.

Pullularia pullulans (CBS 110.67 *Aerobasidium pullulans* (De Bary) Arnoud), was obtained from Centraalbureau voor Schimmelcultures, Baarn. *Aerobacter aerogenes* strains were kindly supplied by Prof. K. Wallenfels, University of Freiburg, and Prof. W.J. Whelan, University of Miami.

Soluble starch was purchased from Merck AG, Darmstadt. Potato starch hydrolyzates were prepared by α-amylase hydrolysis to DE 3.4-10.7, heat inactivation of the enzyme and lyophilization. Pullulan was prepared from *Pullularia pullulans* following the procedure described by Ueda *et al.*[33], and further purified by treatment with cetyltrimethylammonium bromide to remove acidic polysaccharides (I). Low molecular weight pullulan was prepared from this preparation by acid hydrolysis.

Barley β-amylase was obtained from BDH Ltd., Poole. The preparation contained 7.83% protein with a specific activity of 212.4 U/mg. Pullulanase, which is not commercially available, was prepared from *Aerobacter aerogenes* by the method of Wallenfels *et al.*[34], as modified by Mårtensson (II). After extraction of the intracellular enzyme, DEAE-cellulose treatment and lyophilization, the preparation contained 80% protein with specific activities of 4.3-6.8 U/mg.

The immobilized β-amylase and pullulanase (III, IV) contained 48.6 and 1.70 U/mg protein respectively with bound protein amounts of 6.8 and 19 mg/g dry polymer. Corresponding values for the immobilized two-enzyme system (V) were 50.5 and 0.94 U/mg protein with bound protein amounts of 6.8 and 35.3 mg/g dry polymer.

Cultivation of the organisms

Growth of *Pullularia pullulans* for pullulan production was performed in a mineral culture medium containing ammonium sulphate, yeast extract and sucrose as the nitrogen and carbon sources respectively. Pullulanase production by *Aerobacter aerogenes* was optimized by growing the cells in a modified Czapek medium where ammonium sulphate, peptone and a combination of glucose and maltose served as the nitrogen and carbon sources respectively. The main cultivation was in both cases carried out in a laboratory fermentor FL 110, 14 litres capacity, fitted with a LP 300 control unit, purchased from Bio Tec, Bromma.

Analytical methods

Determination of reducing sugars was commonly carried out according to the photometric method of Nelson[35], based on copper reduction in

an alkaline solution. Sometimes the method of dinitrosalicylic acid reduction[36] was used. Total carbohydrate was analyzed by the phenol-sulphuric acid method[37]. The iodine binding power has sometimes been used to calculate the degree of polymerization of short chain amylose[38]. Simultaneous determination of glucose, maltose and maltotriose was performed using gas liquid chromatography[39], and the glucose content was sometimes specifically measured by the glucose oxidase-peroxidase method[40]. A microscale method for determination of phosphorus[41] has also been utilized. Isoelectric focussing was carried out as described by Vesterberg *et al.*[42]. Protein measurements of the free soluble enzymes were performed following the procedure given by Lowry *et al.*[43], while the amounts of bound protein of the immobilized preparations were analyzed according to Spackman *et al.*[44].

Assay of enzymic activities of both free and immobilized enzymes were performed essentially in the same way, following the procedures described by Hobson *et al.*[45] and Abdullah *et al.*[46] for β-amylase and pullulanase, respectively. Incubations were carried out in a 0.5% soluble starch solution in 0.04 M acetate buffer, pH 4.8, at 35°C for the β-amylase determination, and for the pullulanase assay in a 1.0% pullulan solution in 0.04 M citrate-phosphate buffer, pH 5.0, at 30°C. One unit of activity (U) was in both cases defined as the amount of enzyme which liberates one μmole of product per min, under the conditions used.

Immobilization procedures

Immobilization of β-amylase and pullulanase was performed in distilled water or in a 0.1 M citrate-phosphate buffer, by first permitting the enzyme to be adsorbed on to the negatively charged copolymer of acrylamide-acrylic acid, whereupon the coupling reagent, the water soluble carbodiimide, was added. After the reaction (18 hr), the immobilized preparation was washed extensively and lyophilized. In the case of β-amylase coupling was sometimes made in the presence of reduced glutathione or bovine serum albumin (IV), and in case of pullulanase in the presence of pullulan (III). Co-coupling was performed by the successive coupling of β-amylase and pullulanase, in that order (V).

Conversions

Different concentrations, 0.5-15% w/v, of pre-hydrolyzed potato starch, DE 3.4-10.7, in a 0.01 M phosphate buffer, usually at pH 6.0 and 45°C, were used in the conversions. For the determination of the optimal ratio of the two enzymes, pH and temperature, as well as for the determination of kinetic parameters such as activation energy, Michaelis constant, inhibitor constant, and finally for control of the conversion kinetics, batch procedures were used in a well-stirred vessel. Continuous conversions were carried out in stirred tank reactors, 5 x 2.5 cm I.D. and 7 x 4 cm I.D., and in plug flow reactors, ranging from 10 x 1 cm I.D. to 40 x 2.4 cm I.D. The flow rates used were in the range of 7.5-112 ml/hr, and the degree of conversion was calculated from measurements of the reducing power according to Nelson[35].

Operational stability measurements were performed either by permitting the enzymes to convert starch in excess in a batch procedure, or by use of the enzymes in a continuous stirred tank reactor for a certain period, whereupon the residual activities were measured.

RESULTS AND DISCUSSION

Pullulan, a polysaccharide necessary for the enzymic assay (I)

The first requirement for any successful enzyme handling is an adequate assay procedure for the enzymic activity. Although the action of pullulanase on amylopectin can be followed by studying the increase in iodine binding or reducing power, these methods are greatly influenced by, or inseparable from α-1,4-hydrolytic activity. Another polysaccharide, pullulan[47], containing almost quantitatively maltotriose units joined through α-1,6-glucosidic linkages, is not attacked by α- or β-amylase but is split by pullulanase into maltotriose units[48], and is the substrate of preference for assay of this enzyme. Therefore pullulan was prepared from cultures of *Pullularia pullulans* following the growth procedure described by Ueda *et al.*[33]. It was subsequently purified from gel-forming acidic polysaccharides with the aid of a quaternary ammonium compound, a step which was shown to be necessary for a convenient handling of the polysaccharide. By using this technique, we developed a purification procedure, with the aid of which pullulan could be precipitated directly from the culture filtrate in a pure form, without many time-consuming reprecipitations, used in the original purification procedure[47].

Preparation and important properties of the enzymes used (II)

Industrially utilized enzymes are usually prepared in bulk quantities and used in a very crude form, in order to fulfil the requirements of a great heat stability and a moderate price. In contrast, the biochemists tend to prefer pure, crystalline enzymes with a high specific activity, which are usually expensive and less stable with respect to temperature, since they are no longer protected by the presence of other contaminating colloidal material. The purity of an enzyme suitable for use in an immobilized form in a potential industrial process must be a compromise, in order to be able to produce an immobilized preparation with a high catalytic activity per unit weight of carrier, but which at the same time shows good operational stability properties. Furthermore the use of highly purified enzymes is limited for economic reasons. Bearing this in mind, pullulanase has been prepared from a strain of *Aerobacter aerogenes* in a cell-bound form according to a modified method of that described by Wallenfels *et al.*[34]. This was preferred to an extracellular production, which is also possible, in order to avoid precipitation of the enzyme from the culture filtrate by large volumes of organic solvents, resulting in a precipitate which has been reported to be difficult to purify[34,49]. After extraction of the enzyme from the cells with the aid of a surface active agent, it was partially purified by a treatment with DEAE-cellulose (II, table II). We were able to show that by using ammonium sulphate and peptone as the nitrogen source during cultivation of the cells, with simultaneous neutralization of the acid produced, which can be simply performed by incorporating calcium carbonate in the growth medium and by using a cultivation temperature of 28°C, pullulanase could be produced in a good yield (II, table I) compared to the results obtained in other investigations[34,49], where the original growth conditions described by Wallenfels *et al.*[34] were used. No detectable amounts of α-amylase impurities were found in the pullulanase preparation. Although this is of less importance for use in the conversion of starch to maltose, it is a requirement for use of the enzyme in elucidating the structures of certain polysaccharides[11,12]. The pullulanase preparation was shown to have a relatively low isoelectric point (pH 3.5-4.0), which is a characteristic of importance for the immobilization procedure (III, fig. 1).

The β-amylase used in the present investigation was a commercially available preparation, isolated from barley, and of a purity comparable to that of the pullulanase preparation obtained.

Carbodiimide coupling to an acrylic copolymer (III, IV, V)

Of the diffent immobilization methods mentioned in the introduction, a covalent binding procedure has been chosen for the enzymes in the present work, because it is experimentally easy to carry out, it gives an attachment which is not reversed by pH, ionic strength and substrate, the derivative is easy to handle and can be centrifuged or filtered, and finally it can be used in a continuous reactor, giving a product which is not contaminated with the enzyme. The entrapment technique appears less favourable for enzymes with large substrates, which may lead to steric hindrance and considerable diffusional resistance. A host of different water-insoluble natural and synthetic support materials have been used for covalent immobilization. Some of the most frequently used natural polymers, such as agarose, cellulose and dextran, can possibly interact with carbohydrate-splitting enzymes and thus lead to a blocking, due to the high carrier-enzyme affinity, giving a low residual enzymic activity. We have instead chosen a synthetic, commercially available copolymer of acrylamide-acrylic acid for the present purpose. This hydrophilic support is resistant to microbial and enzymic degradation and is an appropriate alternative in situations where a neutral matrix is not a requirement. Furthermore, enzymes can be bound by *e.g.* terminal amino groups of lysine, to the carboxylic groups of the polymer by using a carbodiimide as coupling reagent[50,51] (see Figure 2). In particular, the water-soluble form[51] of this compound is a valuable alternative to other possible coupling reagents because of the simplicity of the procedure, which results in good binding efficiency with high retention of activity. Immobilization procedures for pullulanase and β-amylase have therefore been developed by covalent binding of the individual enzymes to the acrylic support, using a water-soluble carbodiimide.

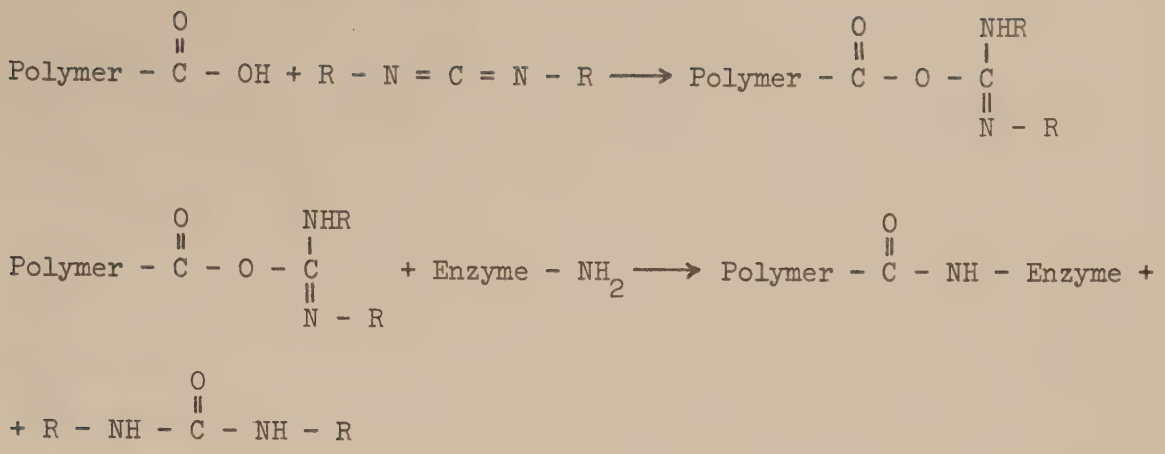

Fig. 2. Carbodiimide coupling of an enzyme to a carboxylic polymer.

Factors affecting the immobilization procedure (III, IV)

Best results were obtained if the enzyme was allowed to be adsorbed on to the gel prior to the coupling. Subsequently the coupling reagent was added, and hence the activation of the acrylic support give the reactive acylisourea, and its further reaction with the enzyme, were performed simultaneously. To remove any non-covalently bound enzyme, washing of the gel beads in an appropriate buffer was shown to be sufficient, although control washings were sometimes performed using solutions of varying pH and ionic strength. We found two factors which in both cases greatly influenced the yield of residual enzymic activity of the immobilized enzyme: the coupling pH and the carbodiimide concentration (III, fig. 2 and IV, fig. 1 and 2). The reason could be that a low pH favours adsorption of the enzyme on to the gel, thus facilitating binding of the enzyme. It is also quite natural that increased amounts of the coupling reagent lead to an increased protein binding. However, the enzyme stability *per se* is decreased when the hydrogen ions and carbodiimide concentrations are increased too much and thus it is necessary for a successful immobilization to establish the optimal conditions. The optimal pH values found for pullulanase and β-amylase were distinct, but in the same range (pH 4.2 and 4.0 respectively), while the optimal carbodiimide concentrations were not so critical and differed between the two enzymes (5 and 15 mg/ml respectively).

The aim of this work has been to prepare immobilized enzyme derivatives showing a high catalytic activity per unit weight of support, although the specific activity of the immobilized preparation would be favoured by lowering the amount of enzyme attached, *e.g.* by decreasing the carbodi-

imide concentration. For immobilized β-amylase, when varying enzyme amounts were used for the coupling procedure, a rather distinct point was obtained, at which an increased amount of enzyme only resulted in a very small increase in the enzymic activity associated (IV, fig. 3). This could be an indication of a limit where the reaction rate begins to be controlled by pore diffusional resistance, or that some steric hindrance of the macromolecular substrate occurs. In any case, the consequence is that a prefered maximum value of the associated β-amylase activity exists, which could possibly be slightly increased, but only at the expense of relatively large quantities of the enzyme.

Enzyme stability during and after immobilization (III, IV)

We found a valuable method of enhancing the enzymic activity of the immobilized enzyme in the case of pullulanase. By incorporating pullulan in the reaction mixture during coupling, the catalytic activity of the enzyme was effectively protected, which resulted in a 5-fold increase in activity over that obtained in the absence of substrate (III, fig. 3). This could also possibly be a consequence of a more favourable steric localization of the enzyme molecules at the matrix surface with the active sites exposed to the outer solution. However, this explanation seems less probable, since the same protective effects were obtained whether the substrate was added before or after adsorption of the enzyme on to the gel. Agents such as cofactors, competitive inhibitors and specific reagents may protect the active site and/or stabilize the active conformation of the enzyme, and should not be forgotten as they may be helpful tools during the immobilization.

By the incorporation of reduced glutathione or bovine serum albumin during the immobilization of β-amylase, which contains certain thiol-groups necessary for its catalytic activity, no increased activity of the immobilized enzyme was obtained. Instead we found that these preparations showed increased operational stabilities compared to the free soluble enzyme, as well as to the normally immobilized preparation (IV, fig. 4). This could be a consequence of a "dilution" of the β-amylase thiol-groups by thiol-groups from the reduced glutathione and bovine serum albumin bound to the matrix, thus lowering the effect of any possible oxidation. The higher protein concentration *per se* could

also be responsible for the increased operational stability, due to a protein-protein interaction.

Immobilization of an enzyme has often, but not always, led to an increased conformational stability of the protein[17,20,21]. If this is a significant characteristic of immobilized enzymes, or if it is a result of the commonly used reporting system where negative results are usually not reported, is not obvious. Stability of an enzyme, free or immobilized, is a function not only of temperature but also of pH, ionic strength and nature of buffer, presence or absence of substrate, protein concentration in the system, time of incubation, and the presence or absence of activators and inhibitors. Data on stability measurements have meaning only when these factors are controlled and stated. However, it is from an industrial point of view usually most interesting to know the enzymic stability under operational conditions when substrate is present. This may be quite different from that obtained otherwise, especially in the case of amylolytic enzymes which are often stabilized by high substrate concentrations. An interesting observation from an academic point of view is, however, that we found no enhanced storage stability for our immobilized pullulanase preparation (III). Increase of the enzyme stability could possibly be expected in this special case since the decrease of pullulanase activity during storage has been suggested to be a result of some proteolytic impurities[49], the action of which in the case of an immobilized preparation would be effectively hindered. Obviously other factors than the presence of possible proteolytic impurities determined the storage stability of our enzyme preparation.

Possible use of immobilized pullulanase to produce short chain amylose

Free soluble pullulanase has been successfully used in the production of low molecular weight amylose from amylopectin[5,6]. In some unpublished experiments, we were able to show that three relatively wide populations of polysaccharide material were obtained by the analysis of a Sephadex fractionated pullulanase hydrolysate of pure potato amylopectin. One, not penetrating the gel, consisted of undebranched pullulanase-limit dextrin of the amylopectin and reached about 20% of the total amount of polysaccharide. The other two peaks consisted of linear chains with an average chain length ($\overline{CL}$) of about 50 and 20 glucose units in amounts of about 30% and 50% respectively. This is in good agreement with the

results obtained by other authors[52,53], although it has been claimed that a quantitative splitting of the branch points in amylopectin is possible by sufficiently increasing the pullulanase concentration[54]. However, when the same procedure was performed using a hydrolysate obtained from immobilized pullulanase, about 50% of undebranched amylopectin was obtained, whereas the peak with $\overline{CL}$ 50 was almost undetectable, and the $\overline{CL}$ 20 peak was not significantly changed. Apparently some steric hindrance occurs for the immobilized pullulanase to penetrate the amylopectin molecule and there set free the B chains in the proposed Meyer structure[55]. In view of this, and the very fast retrogradation rate of short chain amylose, which would probably cause a lot of problems, use of immobilized pullulanase for this special purpose seems less attractive. Free soluble pullulanase or perhaps even better isoamylase, which is not sterically restricted to the same extent[54], seem to be more valuable tools for the conversion of amylopectin to low molecular weight amylose. However, in the hydrolysis of starch to maltose by the use of an immobilized two-enzyme system of β-amylase-pullulanase, these problems would in the author's opinion be eliminated, since the starch in this case is quantitatively converted into small, water-soluble saccharides[7,8,16]. Pullulanase is in this case the debranching enzyme of choice, since it has the capability of hydrolyzing the branch linkages in a β-limit dextrin of amylopectin, while such α-1,6-bonds do not fulfil the structural requirements of isoamylase[56].

Co-coupling of β-amylase-pullulanase in a two-enzyme system (V)

For the conversion of starch to maltose by the combined action of β-amylase and pullulanase, the enzymes can be used either simultaneously or successively. Neither of the two enzymes is, however, able to hydrolyze the substrate to completeness without the aid of the other enzyme, and hence a simultaneous enzymic action is preferable. In this way one enzyme opens the amylopectin structure for the other, thus making a complete degradation possible. The most suitable practical ratio of the two enzymes is greatly influenced by, for example, the enzymic stabilities and the degree of initial hydrolysis of the starch used. An experimental estimation of a suitable ratio of the β-amylase-pullulanase activities was, however, found to be about 5-10:1 (V, fig. 1). Bearing this in mind, an immobilized two-enzyme system of β-amylase-pullulanase was prepared by the successive coupling of β-amylase and

pullulanase in that order, using the coupling conditions previously found optimal for the individual enzymes. The amount of β-amylase used for immobilization was chosen with the guidance of the previously obtained preferable maximum enzyme concentration (discussed above), whereupon the amount of pullulanase was given from the ratio discussed above. Co-coupling of the enzymes has been preferred, although a mixture of the two individually immobilized derivatives could also be used, because of the greater operational stability of immobilized β-amylase found at a high local protein concentration. In this case pullulanase could probably stabilize the β-amylase in the same way as bovine serum albumin and reduced glutathion were previously shown to do (IV, fig. 4). Furthermore the enzymes, although they are not catalyzing consecutive reactions in a proper sense, may facilitate each other's action, thus making a total conversion of a substrate molecule within the microenvironment of one enzyme-gel bead possible, which could favour the kinetics of the reaction.

It was possible, and necessary in order to control the enzymic ratio, to determine the separate enzymic activities of the co-coupled enzyme system by using the original assay procedures, since β-amylase is unable to attack pullulan and has a very low affinity for maltotriose[57], and since the contribution to the initial increase in reducing power from pullulanase, when the β-amylase activity was determined on starch, is negligible at the enzyme concentrations used.

Prerequisites for a kinetic treatment of the immobilized two-enzyme system (VI)

To be able to make an estimation of the industrial potential of any immobilized enzyme system the answers to two major questions must be sought, namely: "What is the efficiency of the immobilized preparation working in an enzyme reactor?" and "How long a period of time is it justifiable to make use of the enzyme?" The answers to these questions are in turn dependent on many process parameters, some of which may be optimized independently of other factors, while the others must be put together in a mathematical relationship. Such a model can then provide the answer to industrially important questions such as: "What is the amount of enzyme required in a continuous reactor to convert starch of a certain initial degree of hydrolysis and concentration, to a certain extent at a given rate?" and "How is the flow rate of product out of an

enzyme reactor influenced by the enzyme instability if a constant degree of conversion is desired?" Models of this type have previously been reported for single-step enzymic reactions where a simple Michaelis-Menten relationship has been assumed for the catalytic reaction[32,58].

In order to formulate such a mathematical model, the kinetics of the enzymic reaction must be considered. An exact kinetic treatment of the immobilized two-enzyme system β-amylase-pullulanase is very complicated since the exact behaviour of the catalytic reaction is not known. Furthermore, in this system the enzyme ratio has been chosen so that none of the enzymes should be strictly rate limiting for the reaction. However, a slight change in the ratio of β-amylase-pullulanase, keeping the amount of β-amylase constant, does not notably affect the reaction rate, as long as the degree of conversion is not close to 100% (V, fig. 1). In the present investigation of the enzyme kinetics, the approximation has therefore been made that β-amylase can be treated as the rate-determining component of the immobilized two-enzyme system. However, this approximation becomes no longer justifiable if the pullulanase activity decreases so much faster than the β-amylase activity during operation that the ratio of β-amylase-pullulanase activity would be increased more than about 2-3 times over the initial ratio. We have shown from operational stability tests that this is not the case (VI, fig. 3-8).

Formulation of a process kinetic model (VI)

Of the many parameters affecting the reaction, the operational pH and temperature were first studied (V, fig. 2 and 3). Although an optimal process pH was found, where the activity as well as the stability of the immobilized system were favoured, the optimal temperature is not so well defined. A change of the latter affects the activity and stability in opposite directions. The preferred temperature is that which gives the best combination of activity and stability during the total time of utilization. We calculated this from a combination of the Arrhenius equation and operational stability measurements (VI, fig. 1-8). The operational stability of both enzymes was in turn found to be dependent on the starch concentration used, *i.e.* high substrate concentrations markedly increased the operational stability

of the enzymes. In practice even other factors influencing the choice of process pH and/or temperature must be considered, such as the risk for microbial contamination and special properties of the substrate or product, for example in this case the starch retrogradation tendency. For this special system a combination of pH 6.0 and 45°C has been chosen, even if the temperature could possibly be raised some degrees if even higher substrate concentrations were used.

With these variables fixed, experiments have been made to determine the kinetic parameters, such as the apparent Michaelis constant (K'_m), the maximum reaction rate and the apparent inhibitor constant of maltose (K'_i), which has been shown to be a competitive inhibitor of β-amylase[59,60]. We also compared these values with those of the free soluble enzyme system, and noticed a slightly increased K'_i and a markedly increased K'_m for the immobilized preparation (VI, fig. 9-12). This reflects the occurence of some diffusional resistance or steric hindrance, which is more pronounced in the case of the macromolecular starch than for the smaller maltose molecules. To establish the extent to which the Michaelis-Menten kinetics can be applied to this process, batch conversions have been made in which we compared the experimental course of the conversion with the predicted one, which was calculated by use of the kinetic data (VI, fig. 13 and 14). It was necessary to set the reaction rate constant (k_3) as a function of the degree of conversion ($k_3 \cdot f(x)$), to obtain a good fit between the experimental conversion and theoretical prediction. This is justifiable since it is known from previous work that β-amylase has much greater affinity for high molecular weight substrates[57]. By use of this kinetic reaction model and a substrate mass balance applicable to the continuous enzyme reactors studied, we were able to formulate theoretical process models for the continuous stirred tank ractor (CSTR) and the plug-flow reactor (PFR).

For the CSTR:

$$E_t = \frac{Q}{k_3 \cdot f(x)} \cdot \frac{1}{\beta} \cdot \frac{(x - x_o)}{(1 - x)} \left[K_m + \frac{342\ K_m}{324\ K_i} S_o (x - x_o) + S_o (1 - x) \right]$$

For the PFR:

$$E_t = \frac{Q}{k_3 \cdot f(x)} \cdot \frac{1}{\beta} \left[K_m \left(1 + \frac{342\, S_o\, (1 - x_o)}{342\, K_i}\right) \ln \frac{1 - x_o}{1 - x} + S_o\, (x - x_o) \cdot \right.$$

$$\left. \cdot \left(1 - \frac{342\, K_m}{324\, K_i}\right) \right]$$

In this equations β is the voidage of the reactor system under consideration.

These models show the relationship between such variables as the amount of enzyme required (E_t), the flow rate of substrate and product through the reactor (Q), the initial degree of hydrolysis (x_o) and concentration of the substrate (S_o), and the finally obtained degree of conversion (x). By using these formulas together with the determined operational stability data, which describe the decay of enzymic activity (E_t) with time (t) at different substrate concentrations (S_o), a relationship between E_t and the different variables involved will take the form:

$$E_t = f\,(t, S_o, Q, x, x_o)$$

With the aid of this information, the important questions raised above can be answered.

Practical conversions of starch to maltose (V, VI)

In order to verify the practical applicability of the formulated theoretical equations, experimental conversions were made under varying conditions, where the results obtained were found to be in good agreement with the theoretical predictions, which indicate that if the reaction rate is controlled by diffusional resistance, it is within the same range in all the experiments (VI, fig. 16 and 17). This is an important conclusion in view of the dramatic reduction of the degree of conversion, due to mass transfer limitations, which has been observed by other authors[57] in certain reactor systems. The composition of the product was shown to be dependent on the initial degree of hydrolysis of the substrate, as well as on the degree of conversion (V, table II), quite in accordance with the theoretical predictions. An increase

in the former made a higher flow rate possible, with constant degree of conversion, and possibly a lower tendency of starch retrogradation was obtained (V, fig. 6). In return the product will contain a relatively higher amount of glucose. To be able to obtain a degree of conversion close to 100%, it was shown that comparatively large amounts of enzyme were required, which limits the use of the reactors in this range. The reactor kinetics were such as to favour a PFR over a CSTR (VI, fig. 15). However, the final choice of reactor system can only be made after considering other aspects, some of which are mentioned in the introduction. In our experimental conversions it was shown to be necessary to use a preservative only at the lowest substrate concentrations. No problems with microbial growth were noticed otherwise, probably due to the higher osmotic pressure obtained when high substrate concentrations were used. A tendency of starch retrogradation in the reactors was noticed at the highest substrate concentration, with the laboratory equipment at hand. This problem could possibly be diminished in a potential large scale process by use of freshly made liquefied starch, perhaps prepared in a "jetcooker" and preferably passed through a filter system before use in the reactor.

An estimation of the economic feasibility of this process cannot be made until more detailed information about use on a larger scale is available. Furthermore, it greatly depends on the raw material costs for the immobilization procedure. More generally, in the author's opinion, immobilized enzymes have for the moment their greatest potential in the production of complex, relatively expensive products, especially in the pharmaceutical industry. However, in certain other cases, where the operational stability of the immobilized enzyme system is sufficiently good, this special technique can be of significant commercial value, even in processes which utilize cheap raw materials and where the degree of refinement is relatively low.

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